Impact of *Morus alba* L. on the expression of drug-metabolizing enzymes and transporters in Caco-2 cells

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**ABSTRACT**

*Morus alba* (MA) L. (mulberry) fruit has been consumed for a long time as a food and a source of vitamin C, anthocyanins, flavonoids, and alkaloids. The effects of MA fruit extract on drug-metabolizing genes and drug transporters were investigated in human colorectal adenocarcinoma cells. Cells treated with MA or its major bioactive constituents, rutin, and cyanidin-3-O-glucoside (C3G), were assessed for cell viability, production of reactive oxygen species (ROS), and alanine aminotransferase (ALT) and aspartate transaminase (AST) levels. The mRNA expression of target metabolic genes was determined using real-time polymerase chain reaction. Cell viability remained higher than 80%, and ROS levels were unchanged by all treatments. MA, C3G, and rutin did not alter the ALT and AST levels or expression of CYP2D6 and sulfotransferase 1A1 (*SULT1A1*). However, MA, C3G, and rutin down-regulated the expression of CYP2C19, CYP3A4, uridine diphosphate-glucuronosyltransferase 1A6 (*UGT1A6*), N-acetyltransferase 1, and organic anion transporting polypeptide 1B1 (*OATP1B1*), and MA elevated the expression of CYP1A2. In addition, MA induced CYP1A2 and CYP2C19 expression in combination with aspirin, caffeine, and simvastatin and upregulated expression of *UGT1A6* and *SULT1A1* in combination with paracetamol. Therefore, consumption of MA fruit or its supplements poses a risk for drug interactions via its modulation of cytochrome P450 and conjugation enzyme-associated metabolism and *OATP1B1*-mediated drug transport.

**INTRODUCTION**

The fruit of *Morus alba* (MA) L. (mulberry), from the family Moraceae, has been consumed as a food and dietary supplement for a long time and is rich in vitamin C, anthocyanins, flavonoids, and alkaloids. MA fruit and fruit extracts have shown antioxidant, anti-diabetic, anti-atherosclerotic, anti-besity, anti-cholesterol, and antibacterial activities, as well as immune enhancement, neuroprotective, and hepatoprotective properties (Chan et al., 2016; Chang et al., 2021; Hansawasdi and Kawabata, 2006; Zafar et al., 2013; Zhang et al., 2018). MA extracts are considered safe to use without severe side effects. MA leaf extract exhibited no acute toxicity in rats at doses above 5 g/kg (Abdulla et al., 2009), but mild itching was reported after topical application of MA oil for melasma treatment (Alvin et al., 2011). MA has been used as a nutraceutical product or food supplement due to the presence of several functional and pharmacological compounds in its leaves, fruit, and seeds, such as mulberroside A, rutin, chlorogenic acid, caffeic acid, quercetin, gallic acid, kaempferol, and apigenin (Chen et al., 2021; Hansawasdi and Kawabata, 2006; Mei et al., 2012).

All orally consumed substances have the potential for inter with medications (Fugh-Berman, 2000). Polypharmacy is an increasingly common event with physicians needing to prescribe multiple medications for patients with multiple conditions. In addition, people have access to a wide range of over-the-counter medications as well as health and dietary supplements. Herb-drug interactions may modulate the pharmacological and toxicological effects of either the herb or the drug. Information about MA herb-drug interactions is very limited.
Cytochrome P450 (CYP) monooxygenase enzymes, such as CYP1A2, CYP2C9, and CYP3A4, are responsible for phase I metabolism of greater than half of all clinical drugs (Zanger and Schwab, 2013). Foods or supplements can affect the metabolism of clinical drugs by interfering with the regulatory expression of CYPs. Conjugation enzymes involved in hepatic phase II metabolism including uridine diphosphate- (UDP-) glucuronosyltransferase 1A6 (UGT1A6), N-acetyltransferase 1 (NAT1), and sulfotransferase 1A1 (SULT1A1) can also play an important role in the metabolism of a variety of clinical drugs, xenobiotics, and toxicants (Kim et al., 2019; Rasool et al., 2019; Witham et al., 2017). In addition to phaseaction I and II metabolizing enzymes, some drug transporters such as organic anion transporting polypeptide 1B1 (OATP1B1) and ATP-binding cassette (ABC) transporter protein 1 (ABCB1 or P-glycoprotein) are important determinants for transporter-mediated drug interactions (Chen et al., 2019; Kayesh et al., 2021; Meszaros et al., 2013; Sort et al., 2018).

A monolayer of human colorectal adenocarcinoma cells (Caco-2) is the most suitable in vitro model representing the human intestine for pharmacokinetic studies, especially for studies of the bioactivity of foods and herbal supplements (Brück et al., 2017; Ifitikhar et al., 2020; Vaessen et al., 2017). The reduction of oxygen during normal biochemical or metabolic reactions in living cells or organisms results in the production of intermediate metabolic byproducts known as reactive oxygen species (ROS), which can cause oxidative damage to cells or tissues (Kükürt et al., 2021). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzyme markers of oxidative damage to hepatocytes but can also be released by other damaged cell types (Sepulveda, 2019; Udomsak et al., 2022). Hence, this study investigated the effects of MA on ROS, AST, ALT, and the mRNA expression of several important phases I and II drug-metabolizing enzymes and drug transporters in a Caco-2 monolayer model.

MATERIALS AND METHODS

Reagents

Cyanidin-3-O-glucoside (C3G) and rutin were supplied by Chengdu Alfa Biotechnology Co., Ltd. (Chengdu, China). High-performance liquid chromatography (HPLC) solvents were products of RCI Labscan Ltd. (Bangkok, Thailand). Dulbecco’s modified Eagle medium (DMEM) (+) phenol red, DMEM/F12 phenol red-free medium, Dulbecco’s phosphate-buffered saline, fetal bovine serum (FBS), 1× penicillin, streptomycin, and neomycin antibiotics (PSN), and 1× GlutaMAX® were supplied by Gibco® (Thermo Fisher Scientific, MA). Resazurin, 2,4-dinitrophenylhydrazine (DNPH), 2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA), rifampicin, and ketoconazole, L-aspartate, L-alanine, and α-ketoglutarate were products of Sigma-Aldrich (Missouri). ReverTra Ace®, random primers, and other reagents for reverse transcription were products of Toyobo Co., Ltd. (Osaka, Japan). Primers were synthesized by Bio Basic, Inc. (Markham, Ontario, CA). Taq polymerase, RNase inhibitor, deoxynucleotide triphosphate mixture, and other reagents for polymerase chain reaction (qPCR) were products of Vivantis Technologies Sdn. Bhd. (Selangor, Malaysia). Other laboratory chemicals were obtained from commercial suppliers with the highest purity and quality.

Preparation of MA fruit powder

Seedlings of MA L. cultivar Chiangmai were provided by the Queen Sirikit Department of Sericulture, Ministry of Agriculture and Cooperatives, Thailand, and were grown in Noen-Ruang Village, Muang District, Khon Kaen, Thailand. The fruits of MA were collected in March 2021. A plant specimen (Panya, T. 8 KKU No. 25978) was deposited at the Research Institute for Human High Performance and Health Promotion, Khon Kaen University. The MA fruits were washed in cold water three times and dried in a hot air oven at 50°C until the moisture content was 10%. The dried fruits were mashed and sieved (60 mesh) before storage in a vacuum aluminum package until further analysis.

HPLC analysis

MA fruit powder, C3G, and rutin were accurately weighed and dissolved in absolute methanol before being filtered using a 0.45 μm membrane. The C3G and rutin contents of MA fruit powder were analyzed by HPLC-UV. A Hypersil ODS (4.0 × 250 mm × 5 μm, Agilent Technologies Inc., Santa Clara, CA) column was used as the stationary phase. A gradient mobile phase of acetonitrile in 0.1% phosphoric acid was increased from 5% to 80% in 25 minutes with a flow rate of 1 ml/minute. The machine (Prominence-i Model LC-2030C 3D, Shimadzu Corporation, Kyoto, Japan) was set up with dual UV detectors at 517 nm for C3G and 365 nm for rutin. The HPLC method validation and the chromatogram are shown in Table 1 and Figure 1.

Cell culture

Caco-2 cells (RBRC-RBC0988) were supplied by the Cell Bank of RIKEN BioResource Center (Saitama, Japan). The cells were maintained in DMEM (1 g/l D-glucose) with 20% FBS, L-glutamine, sodium pyruvate, 1× GlutaMAX®, and 1× PSN at 37°C with 5% CO₂ and 95% relative humidity. The cells were cultured as a monolayer in 24-well plates (2.5 × 10⁵ cells/well) at 37°C for 30 minutes. The resorufin formation was measured for spectrofluorometric intensity at excitation of 530 nm and emission of 580 nm. The percentage of cell viability was measured for the resazurin assay (Alamar Blue assay) as previously described (Sriset et al., 2021). Briefly, the living cells were incubated with 100 μM resazurin in 5% CO₂ at 37°C for 30 minutes. The resorufin formation was measured for spectrofluorometric intensity at excitation of 530 nm and emission of 580 nm. The percentage of cell viability was...
calculated from an increasing rate of resorufin compared to the nontreatment group.

### Determination of ROS

The ROS level in the cell culture medium was measured using the DCFH-DA reaction as previously described (Sriset et al., 2021). The medium was incubated in the dark with 62.5 nM DCFH-DA for 40 minutes. The fluorescence intensity of 2′,7′-dichlorofluorescein was measured at excitation and emission wavelengths of 484 and 530 nm, respectively, using a spectrofluorometer. The ROS level was calculated by comparison with a standard curve of hydrogen peroxide (2.5 to 20 μM).

### Determination of cellular transaminases (AST and ALT)

An ALT substrate (300 mM L-alanine and 0.7 mM α-ketoglutarate) or AST substrate (10 mM L-aspartate and 1.7 mM α-ketoglutarate) was incubated with a cell culture medium or sodium pyruvate standard solution at 37°C for 20–30 minutes. Then, 5 mM DNPH was added, and the mixture was left at room temperature for 20 minutes. The reaction was stopped by the addition of 1.3 N sodium hydroxide. The absorbance of the mixture was measured at 505 nm. The levels of AST and ALT were determined as international units per liter (IU/l) by comparison with a standard curve of sodium pyruvate (Sriset et al., 2021).

### Quantitative determination of mRNA expression by reverse transcription (RT/qPCR)

At 48 hours after treatment, Caco-2 cells were collected using a guanidinium thiocyanate-phenol solution. Chloroform was added to extract RNA. The RNA was precipitated by isopropanol, and the pellet was washed twice with cold 70% ethanol before measuring the concentration, purity, and integrity of the RNA. Total RNA (500 ng per reaction) was reverse-transcribed using a ReverTra Ace® kit. The cDNA (20 ng per reaction) was used to determine the expression of target genes with specific primers (Table 2) using RT/qPCR with SYBR Green I probe detection. The expression of target genes was normalized to the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative fold expression was calculated using the ∆∆Ct method.

### Statistical analysis

All results are presented as mean ± standard deviation (SD) from 5 to 6 samples per group. Statistical differences were analyzed using one-way analysis of variances coupled with Tukey’s procedure using IBM-SPSS version 23 software (Armonk, NY). p ≤ 0.05 was considered statistically significant.

### RESULTS AND DISCUSSION

#### The effect of MA fruit on cell viability, ROS, AST, and ALT

Phytochemical compounds including rutin, apigenin, chlorogenic acid, kaempferol, umbelliferone, morin, and luteolin have been isolated from the leaves, fruit, branches, bark, and roots of MA (Chu et al., 2006). Mulberroside A is a bioactive constituent previously found in the ethanolic extract of MA root (Park et al., 2011). In this study, we determined the mulberroside A, C3G, rutin, ellagic acid, chlorogenic acid, quercetin, kaempferol, galangin, genistein, luteolin, morin, myricetin, hesperidin, and silymarin contents of MA fruit powder, using HPLC (data not shown). The HPLC chromatogram (Fig. 1) indicates that rutin and C3G are the major constituents of MA. The rutin and C3G contents of MA were 7.85% ± 0.29% and 7.65% ± 0.31%, respectively (Table 1). These observations were consistent with a previous study that showed mulberry fruit contained a large amount of anthocyanins, which were mainly composed of cyanidin 3-rutinoside and C3G (Du et al., 2008). Rutin has been found in many parts of MA, including the leaves, fruit, and roots (Hunyadi et al., 2012; Zhao et al., 2015). The amount of these constituents can vary depending on the plant source, the harvest period, the method of extraction,

### Table 1. Reversed phase HPLC validation and rutin and C3G contents in MA extract.

<table>
<thead>
<tr>
<th></th>
<th>Rutin</th>
<th>C3G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specificity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>λ_max (nm)</td>
<td>365</td>
<td>517</td>
</tr>
<tr>
<td>Retention time</td>
<td>14.1</td>
<td>13.5</td>
</tr>
<tr>
<td>Linearity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration range (µg/ml)</td>
<td>20 - 120</td>
<td>20 - 120</td>
</tr>
<tr>
<td>Linear regression equation</td>
<td>( y = 27,698x - 72,894 )</td>
<td>( y = 36,529x - 23,590 )</td>
</tr>
<tr>
<td>( R^2 )</td>
<td>0.9988</td>
<td>0.9992</td>
</tr>
<tr>
<td>Accuracy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Recovery ± % RSD</td>
<td>100.41 ± 1.09</td>
<td>100.28 ± 1.02</td>
</tr>
<tr>
<td>Precision (% RSD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within day</td>
<td>0.29–1.47</td>
<td>0.28–1.34</td>
</tr>
<tr>
<td>Between day</td>
<td>0.56–1.34</td>
<td>0.63–1.82</td>
</tr>
<tr>
<td>Sensitivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>5.76</td>
<td>3.51</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>17.47</td>
<td>10.65</td>
</tr>
<tr>
<td>% Content in MA (± SD)</td>
<td>7.85 ± 0.29</td>
<td>7.65 ± 0.31</td>
</tr>
</tbody>
</table>
Since high levels of rutin and C3G were found in the MA sample, experiments in Caco-2 cells were continued with these two compounds. Previously, Zhang et al. (2013) examined the effect of 5 to 200 µM rutin on absorption and metabolism in Caco-2 cells (Zhang et al., 2013), and Ferrari et al. (2016) examined the anti-inflammatory and antioxidant activities of 20 to 40 µM C3G in Caco-2 cells (Ferrari et al., 2016). The doses of rutin and C3G (1 and 10 µM) and MA (125 to 500 µg/ml) employed in the current study were chosen to avoid cytotoxic effects and morphological changes to the Caco-2 cells while still determining their effects on the expression of metabolizing enzymes and transporters.

Although Rif, rutin (10 µM), and MA (125 µg/ml) significantly reduced cell viability (Fig. 2A), the cell viability remained above 80%, which enabled us to continue the experiments, especially as MA at higher doses (250 and 500 µg/ml) did not show toxicity. All treatments did not change the ROS level (Fig. 2B). Excessive ROS production occurs in tumor cells, including Caco-2 cells. Rutin (5–100 µM) has been shown to possess ROS scavenger activity (ben Sghaier et al., 2016), and C3G (20–40 µM) improved intracellular redox status through...
inhibition of NF-κB signaling (Ferrari et al., 2016), in Caco-2 cells. Moreover, phytochemical-rich MA has been shown to have antioxidant and anti-inflammatory capacities (Gryn-Rynko et al., 2016). Cellular transaminases (ALT and AST) are common markers of cell injury (Pandurangan and Kim, 2015; Sriset et al., 2021). Keto and Rif markedly elevated levels of AST (Fig. 2C) and ALT (Fig. 2D) in Caco-2 cells, while all other treatments did not. Keto and Rif (at 10 µM) have also been shown to modify the expression of CYPs and transporters in Caco-2 cells (Netsch et al., 2006; Takano et al., 1998). As MA did not show toxicity in Caco-2 cells, all tested compounds were employed using these concentrations.

**Alteration of phase I CYP expression by MA fruit**

*CYPIA2* is constitutively expressed in the human liver and is detectable in other tissues and cell lines such as Caco-2 cells (Netsch et al., 2006; Zanger and Schwab, 2013). *CYPIA2* is responsible for the metabolism of antidepressants and antipsychotics, as well as anti-inflammatory, anesthetic, and analgesic drugs (Zhou et al., 2010). Keto extensively induced expression of *CYPIA2* mRNA (*p* < 0.001), while Rif, C3G, and rutin did not (Fig. 3A). All doses of MA markedly elevated *CYPIA2* expression.

*CYPIA2* is responsible for the metabolism of proton pump inhibitors (e.g., omeprazole, esomeprazole, and pantoprazole), antidepressants (e.g., imipramine, amitriptyline, sertraline, and fluoxetine), hypnotics and sedatives (e.g., diazepam, clobazam, and phenobarbital), phenytoin, proguganil, nelfinavir, voriconazole, and clopidogrel (Uppugunduri et al., 2012). *CYP2C19* is inducible by several drugs such as rifampicin, dexamethasone, and phenobarbital (Rana et al., 2010). Rif significantly induced expression of *CYP2C19* mRNA, while Keto, C3G, rutin, and MA strongly suppressed *CYP2C19* (Fig. 3B). Rif has previously been shown to increase *CYP2C19* activity in human enterocytes (Glaeser et al., 2005). On the other hand, Keto has been reported as a *CYP2C19* inhibitor (Uppugunduri et al., 2012).

*CYP2D6* plays an important role in the liver and brain with reg to the metabolism of drugs such as beta-blockers, opiates, antiarrhythmic agents, neuroleptics, and antidepressants as well as neurotransmitters (Darney et al., 2021). The expression of *CYP2D6* mRNA was unchanged by any of the treatments (Fig. 3C).

The most important metabolizing enzyme in phase I is *CYP3A4* which metabolizes a large and diverse range of molecules covering more than half of the clinical drugs, including antihypertensive, antibacterial, antifungal, and antiviral drugs, bronchodilators, and lipid-lowering agents (Zanger and Schwab, 2013). Rif significantly increased the expression of *CYP3A4* mRNA, while Keto, C3G, and MA suppressed *CYP3A4* expression. Rutin did not affect *CYP3A4* expression (Fig. 3D).

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**Table 2. Specific primers for RT/qPCR.**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward and reverse primers (5' to 3')</th>
<th>Product size (bp)</th>
<th>Annealing temp (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>F: ACAAGGGACACACGCCTGAA</td>
<td>160</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>R: AGGCTTGTTAATGGGAGTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: GGAUTGTAAGCCACCCCTTG</td>
<td>174</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>R: TAAAGTCCCAGGGGTGTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>F: AGCTTTTCTGTTGACCCCCCCATC</td>
<td>135</td>
<td>61.1</td>
</tr>
<tr>
<td></td>
<td>R: GAGCCCGAGTGGAGAATACTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: CTTCATTCAATGGAATGCTGTA</td>
<td>87</td>
<td>55.0</td>
</tr>
<tr>
<td></td>
<td>R: TCCCAATATAACCTCTACACACACAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4</td>
<td>F: AGGCCAGACCTGTCCTGTA</td>
<td>76</td>
<td>58.2</td>
</tr>
<tr>
<td></td>
<td>R: CCACCTGTTGGAGAAAAGTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAT1</td>
<td>F: GAATCAAGCCAGGAAGAGCA</td>
<td>151</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>R: TCAAGTGCTAATGTCTGCTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SULT1A1</td>
<td>F: GTCCAGGAGTCCCATCTCT</td>
<td>76</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>R: GTCTCCATCCCTGAGGGATAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OATP1B1</td>
<td>F: GAATGCCCCAGAGATGATGCTT</td>
<td>154</td>
<td>60.0</td>
</tr>
<tr>
<td></td>
<td>R: ACCCATGCAAGGATTTCTGAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: GGGATGGTCAGTTGATGGA</td>
<td>110</td>
<td>60.0</td>
</tr>
<tr>
<td>ABCB1</td>
<td>R: GCTATCGTGGTGGCAAAACAAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>F: CACCATCTCCAGAGGGAGGAG</td>
<td>72</td>
<td>61.1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>R: GACTCCACACGTACTGAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. Effects of MA fruit, C3G, and rutin on cell viability, ROS, and cellular transaminases (AST and ALT) levels in Caco-2 cells. *p < 0.05, **p < 0.001 versus control (DMSO). Keto, 10 µM ketoconazole; Rif, 10 µM rifampicin; C3G, 1 and 10 µM C3G; Rutin, 1 and 10 µM rutin; MA, 125, 250, and 500 µg/ml MA fruit (n = 4–5).

Figure 3. Effects of MA fruit, C3G, and rutin on CYP mRNA expression in Caco-2 cells. *p < 0.05, **p < 0.001 versus control (DMSO). Keto, 10 µM ketoconazole; Rif, 10 µM rifampicin; C3G, 1 and 10 µM C3G; Rutin, 1 and 10 µM rutin; MA, 125, 250, and 500 µg/ml MA fruit (n = 4–5).
Keto has been proposed to induce CYP1A2 via activation of the aryl hydrocarbon receptor and pregnane X receptor (PXR) (Novotna et al., 2014). Moreover, Keto was reported as an inhibitor for CYP2C19 and transporters (Nikulin et al., 2017), while Rif was noted as a typical CYP3A4 inducer (Kunchareonwit et al., 2020). Only a few studies have examined the effect of MA and its constituents on the expression of CYPs. However, anthocyanins (including C3G) have been shown to inhibit CYP3A4 enzyme activity in human liver microsomes (Srovnalova et al., 2014), and an in silico analysis showed that rutin (quercetin-3-O-rutinoside) is metabolized by CYP1A2 and CYP2C9 (Sousa et al., 2013). More recently, Kar et al. (2015) performed an in vitro study focusing on the effect of different parts of MA on human recombinant CYP enzymes. While this approach has limitations for the study of induction, MA leaf extract inhibited the activities of human recombinant CYP3A4, CYP2D6, CYP2C9, and CYP1A2 (Kar et al., 2015), which corresponds to the present findings for CYP3A4 and CYP2C19. Therefore, potential MA-drug interactions could occur via CYP1A2 induction and/or CYP2C9 and CYP3A4 suppression.

**Alteration of phase II conjugation enzyme and transporter expression by MA fruit**

The conjugation reactions of phase II metabolism are also keys to the biotransformation of drugs. UGT1A6 is responsible for the biotransformation of a variety of drugs such as aspirin, carvedilol, naproxen, valproic acid, and zidovudine (Kim et al., 2019). In the present study, Rif extensively induced expression of UGT1A6 mRNA, while Keto did not (Fig. 4A). In contrast, C3G, rutin, and MA significantly suppressed UGT1A6 expression. These results correspond with previous observations that Rif induced UGT1A6 expression in the human colon (van de Kerkhof et al., 2008), and morusin, a prenylated flavonoid isolated from the root bark of MA, inhibited expression of UGT1A6, UGT1A7, and UGT1A8 in human, rat, dog, monkey, and minipig liver microsomes (Shi et al., 2016). Hence, MA and its constituents (C3G and rutin) possess the potential to downregulate UGT1A6 expression in Caco-2 cells.

NAT1 is responsible for the metabolism of nitrogenous compounds such as aromatic amines/amides, hydrazine, and hydrazides and is also involved in the biotransformation, clearance, and toxicity of numerous pharmacologic agents and environmental toxicants (Walker et al., 2009). Rif, C3G, and MA suppressed expression of NAT1, while Keto and rutin did not show any significant change (Fig. 4B). Information about the effect of MA, C3G, and rutin on NAT1 is limited, and this is the first report of the inhibitory effects of MA and C3G on NAT1 expression in Caco-2 cells.

SULT1A1 is the key enzyme responsible for the sulfation of xenobiotics, including paracetamol, estrogens, and iodothyronines (Gamage et al., 2005; Rasool et al., 2019). Only Rif significantly induced the expression of SULT1A1, while the other tested compounds did not affect SULT1A1 expression (Fig. 4C). Previously, the expression of SULT2A1 mRNA was shown to be up-regulated by rifampicin in human hepatocytes (Fang et al., 2007). This might correlate with the observation that Rif induced SULT1A1 expression in Caco-2 cells.

OATP1B1 has been reported to be associated with the uptake transport of clinical drugs, including statins, antibiotics, and anticancer drugs (Zhang et al., 2007). Keto, Rif, C3G, and rutin at 10 µM and all doses of MA significantly suppressed the expression of OATP1B1 (Fig. 4D). The downregulation of OATP1B1 expression by Keto and Rif in the present study confirms that these two compounds are inhibitors for OATPs (Choi et al., 2011; Pahwa et al., 2017). Similarly, flavonoids, quercetin, and rutin have previously been reported to inhibit the uptake of OATP1B1 substrates (Wang et al., 2005).

ABCB1, the efflux transporter, also known as P-glycoprotein, regulates the plasma and intracellular concentrations of numerous xenobiotics (Gow et al., 2008). Keto, Rif, and C3G all slightly lowered the expression of ABCB1 (although this was not statistically significant), and rutin did not affect ABCB1 expression. The two higher tested doses of MA (250 and 500 µg/ml) suppressed ABCB1 expression (Fig. 4E). This was consistent with a previous study that showed MA root extract suppressed ABCB1 expression in human breast cancer MCF7 cells (Choi et al., 2013).

In summary, MA downregulated the expression of UGT1A6, NAT1, OATP1B1, and ABCB1. Therefore, MA fruit might cause drug interactions by disrupting of phase II biotransformation and drug transport via those related genes.

**Effect of combinations of MA fruit and common drugs on phase I CYP expression**

People are exposed to many different substances in their daily life that can affect phase I and II drug-metabolizing enzymes and transporters, especially through the consumption of commonly prescribed and over-the-counter medications. Paracetamol (acetaminophen or APAP) is a commonly used analgesic and anti-pyretic drug that is available in drugstores as an over-the-counter medication. APAP is mainly metabolized by CYP2E1, CYP1A2, and CYP3A4, and a metabolite of APAP, N-acetyl-p-benzoquinoneimine, can cause hepatotoxicity (Toes et al., 2005). Caffeine (Caf) is a bioactive component found in coffee, tea, soft drinks, and energy drinks. Although the daily intake of a cup of coffee, which contains 400 mg of Caf, is safe for health, caffeine can cause significant clinical pharmacokinetic interactions with many drugs (Belayneh and Molla, 2020). Low-dose aspirin (ASA) is recommended for the prevention of atherothrombotic and cardiovascular events. However, the antiplatelet action of ASA-induced drug interactions has high clinical importance (Saxena et al., 2013). Simvastatin (Sim) is a first-line antilipidemic drug commonly prescribed to reduce the risk of cardiovascular events, but it can also cause a variety of serious adverse effects such as myalgia, myopathy, and hepatotoxicity. Several drugs and compounds have been reported to disturb the metabolism of Sim, leading to an increase in the risk of these adverse effects (Kellick et al., 2014). Therefore, we examined the effect of MA combined with four commonly encountered compounds, APAP, ASA, Caf, and Sim, on profiles of CYPs and related genes in Caco-2 cells. On their own, APAP, ASA, Caf, and Sim did not change the expression of CYP1A2 or CYP2C19 mRNA, but when combined with MA, each of ASA, Caf, and Sim synergistically increased the expression of CYP1A2 and CYP2C19 mRNA.
Although MA also induced CYP1A2 on its own, it had the opposite effect on CYP2C19, inhibiting the expression (Fig. 3B). The expression of CYP2D6 mRNA was not altered by all treatments, either as single compounds or in combination with MA (Fig. 5C). APAP and ASA did not change the expression of CYP3A4 mRNA (Fig. 5D) either on their own or in combination with MA. The treatment of Caco-2 cells with Caf both alone and in combination with MA suppressed the expression of CYP3A4 mRNA to similar levels, while the combination of Sim with the highest dose of MA (500 µg/ml) suppressed the expression of CYP3A4 mRNA (Fig. 5D). In a previous study, the Chinese traditional medicine formulation BuChang NaoXinTong, which contains MA twig and some other herbs, was shown to induce the expression of CYP2C19 in human primary hepatocytes and HepG2 cells via activation of PXR (Sun et al., 2016). Similarly, an aqueous extract of mulberry leaves increased CYP3A4 activity, while an ethanol extract of mulberry leaves reduced CYP1A2, CYP2D6, and CYP2C19 activity in a rat model (Sheng et al., 2021). Therefore, there are several other factors such as the part of the MA plant being used, the extraction method employed, and the combination of MA with other compounds and plants that could affect the regulatory profiles of CYP expression.

Effect of combinations of MA fruit and common drugs on phase II conjugation enzymes and transporters

UGT1A6 is an important member of the human UGT1 family expressed in the liver and extrahepatic tissues, including the intestine, that biotransforms planar phenols and arylamines...
It is responsible for APAP-glucuronide conjugation (Zhao and Pickering, 2011). When tested on their own, both APAP and ASA induced the expression of UGT1A6 mRNA (Fig. 6A) while MA suppressed UGT1A6 expression (Fig. 4A). In combination, MA and APAP showed increased induction of UGT1A6 expression (Fig. 6A). On the other hand, the combination of MA with ASA, Caf, and Sim resulted in the inhibition of the expression of UGT1A6. At present, there is limited information about the effect of MA on UGTs. One report found that morusin, a flavonoid isolated from the root bark of MA, inhibited UGTs in liver microsomes of humans, rats, dogs, monkeys, and minipigs (Shi et al., 2016).

NAT1 is responsible for the metabolism of pharmacological agents and environmental toxicants
All treatments, including singly applied APAP, ASA, Caf, and Sim and their combinations with MA, extensively suppressed expression of NAT1 (Fig. 6B).

SULT1A1 is responsible for the sulfation of APAP (Rasool et al., 2019). APAP significantly elevated the expression of SULT1A1 mRNA (Fig. 6C), while ASA, Caf, and Sim did not. Interestingly, ASA, Caf, and Sim in combination with MA significantly induced SULT1A1 expression.

OATP1B1 transports clinical drugs into cells (Zhang et al., 2007). Flavonoids have been reported to inhibit the uptake of OATP1B1 substrates (Wang et al., 2005). APAP, ASA, Caf, Sim, APAP+MA, and ASA+MA all markedly suppressed OATP1B1 expression, while Caf+MA and Sim+MA controversially showed synergistic induction of OATP1B1 expression (Fig. 7A). The ethanol extract of MA root was reported to downregulate ABCB1 expression in MCF7 cells (Choi et al., 2013).

ABCB1 is involved in the intestinal absorption of aspirin (Li et al., 2017). ASA significantly suppressed the expression of ABCB1 mRNA (Fig. 7B). However, ASA in combination with the highest dose of MA strongly induced the expression of ABCB1 mRNA in Caco-2 cells. To date, information about the effect of MA on drug transporters is very limited. It would be interesting to unravel the effect of MA and its constituents on the expression and activity of other drug transporters.
CONCLUSION

MA fruit, C3G, and rutin did not alter the expression of CYP2D6 and SULT1A1 in Caco-2 cells. Nevertheless, they down-regulated the expression of CYP2C19, CYP3A4, UGT1A6, NAT1, and OATP1B1, and MA upregulated the expression of CYP1A2. The combination of MA with aspirin, caffeine, or simvastatin showed additional induction of CYP1A2 and CYP2C19, while the combination of MA and paracetamol synergistically upregulated the expression of UGT1A6 and SULT1A1. Therefore, the consumption of MA fruit or mulberry supplements poses a risk for drug interactions via the modulation of CYP and conjugation enzyme-associated metabolism and OATP1B1- or ABCB1-mediated drug transport in the human intestine.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit it to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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